

4216.260-US

PATENT

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of: Royer et al.

Serial No.: 09/461,537

Group Art Unit: 1636

Filed: December 15, 1999

Examiner: I. Yucel

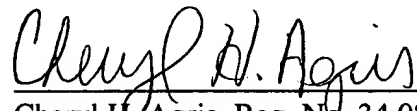
For: NON-TOXIC, NON-TOXIGENIC, NON-PATHOGENIC *FUSARIUM*  
EXPRESSION SYSTEM AND PROMOTERS AND TERMINATORS FOR USE  
THEREIN

**COMMUNICATION**

Attached hereto is a Declaration by one of the inventors, Wendy T. Yoder, showing comparative data of heterologous gene expression of *Fusarium venenatum* ATCC 20334 with other *F. venenatum* strains and other *Fusarium* species. Applicants also attach hereto accompanying Figures 1, 2, 3a-3c, 4a-4c, Tables 1-2 and Appendices A-J.

Respectfully submitted,

Date: 5/15/00



Cheryl H. Agris, Reg. No. 34,086

Counsel for

Novo Nordisk of North America, Inc.

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In re Application of: Royer *et al.*

Serial No.: 09/461,537

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For: Non-Toxic, Non-Toxigenic, Non-Pathogenic *Fusarium* Expression System and Promoters and Terminators for Use Therein

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents

Washington, DC 20231

Sir:

I, Wendy T. Yoder, do hereby state and declare that

1. I am one of the inventors of the subject matter disclosed and claimed in the above-captioned application.
2. I received a Ph.D. in Fungal Ecology and Genetics from the University of Bath, United Kingdom, in 1982. I have been employed at Novo Nordisk Biotech, Inc., Davis, California, since 1992 where I am currently a Senior Scientist.
3. I understand that the Examiner has requested comparative data of heterologous gene expression of *Fusarium venenatum* ATCC 20334 with other *F. venenatum* strains. I also understand that the Examiner has requested that a method be provided to distinguish unambiguously *F. venenatum* from other *Fusarium* species.
4. The experimental study and results described below were performed by me or under my direct control and supervision.

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5. In the experimental study to provide comparative data of expression of heterologous proteins of *Fusarium venenatum* ATCC 20334 with other *F. venenatum* strains, the *Fusarium* strains used were *F. venenatum* ATCC 20334 (=A 3/5), *F. venenatum* BBA 64537, and *F. venenatum* ATCC 60879. Expression vectors were constructed according to Royer *et al.*, 1995, *Bio/Technology* 13: 1479-1483 containing *Humicola insolens* cellulase gene (CAREZYME™) designated pDM151, or *Thermomyces lanuginosus* lipase gene (LIPOLASE™) designated pDM155, using the *Fusarium oxysporum* trypsin gene (SP387) promoter and terminator for expression of the genes (U.S. Patent No. 5,837,847) and the *Aspergillus nidulans amdS* gene as the selectable marker. See Figure 1 for restriction maps of these vectors.

Protoplasts of the *F. venenatum* strains were prepared from young germlings, as follows: Fernbach flasks containing 500 ml of RA medium (succinic acid (disodium salt) 50 g/l, 50 X Vogels salts 20 ml/l, NaNO<sub>3</sub> 12.1 g/l, glucose 1 g/l) were inoculated with 10 plugs from an agar plate and incubated at 24°C, 150 rpm for 32 hours. Spores were harvested through Miracloth (Calbiochem, San Diego, CA) and centrifuged for 20 minutes at 7000 rpm in a Sorvall RC-5B centrifuge (E. I. DuPont De Nemours and Co., Wilmington, DE). Pelleted spores were washed twice with sterile distilled water, and then resuspended in sterile distilled water at a concentration of 5 X 10<sup>7</sup>/ml.

One hundred ml of YPG medium (yeast extract 10 g/l, BACTO peptone 20 g/l, glucose 50 g/l) were inoculated with 1 X 10<sup>8</sup> freshly-generated spores of each strain and incubated for 15 hours at 24°C and 150 rpm. The cultures were centrifuged for 7 minutes at 3500 rpm in a Sorvall RT 6000D. Pellets were washed twice in 30 ml of 1 M MgSO<sub>4</sub> and resuspended in 20 ml of 5 mg/ml of Novozyme 234 (Novo Nordisk A/S, Bagsværd, Denmark) in 1 M MgSO<sub>4</sub>. Cultures were incubated at 24°C and 150 rpm for 30 – 60 minutes, then washed once with 2 M sorbitol and twice with STC (0.8 M sorbitol, 25 mM Tris pH 8, 50 mM CaCl<sub>2</sub>). Protoplasts were resuspended in a 9:1:0.1 solution of STC:SPTC:DMSO to a final concentration of 2 X 10<sup>7</sup> protoplasts/ml and stored at -80°C, after controlled-rate freezing in a Nalgene Cryo 1°C Freezing Container (VWR Scientific, Inc., San Francisco). SPTC is composed of 40% PEG 4000, 0.8 M sorbitol, 25 mM Tris pH 8, 25 mM CaCl<sub>2</sub>.

Each transformation reaction comprised 100 µg of pDM153 or pDM151 DNA in 60 µl of 10 mM Tris pH 8.0, 2 ml of protoplasts (at a concentration of  $2 \times 10^7$  per ml) and 50 µl of 5 mg/ml heparin in STC. Plating of the transformation reactions was as described in Royer *et al.*, 1995, *supra*.

The total number of transformants obtained per strain per plasmid (pDM151 and pDM155, respectively) were: *F. venenatum* ATCC 20334= 39, 51; *F. venenatum* BBA 64537= 51, 67; and *F. venenatum* ATCC 60879= 29, 27.

A positive control strain was also run for each gene above. The control strains were *F. venenatum* JRoy30.3 containing pJRoy30 for CAREZYME™, and *F. venenatum* DM194.19.2 containing pDM194 for LIPOLASE™. Vectors pJRoy30 and pDM194 contain the *Fusarium oxysporum* trypsin gene (SP387) promoter and terminator for expression of the genes and the *bar* gene as the selectable marker. See Figure 2 for restriction maps of these vectors.

Agar plugs of each transformant were inoculated into 125 ml baffled glass shake flasks containing 25 ml of medium suitable for expression of the genes and incubated at 28°C and 200 rpm for 7 days. Centrifuged supernatants from each culture were assayed for enzyme activity as described below.

CAREZYME™ activity was determined at 600 nm using 2% azo-carboxymethylcellulose as substrate in 100 mM MOPS pH 7.0 buffer at 45°C for 30 minutes. The activity was calculated in reference to a CAREZYME™ standard curve generated by linear regression using concentrations of 20,15,10,5 and 2.5 ECU/ml.

LIPOLASE™ activity was determined at 405 nm using p-nitrophenyl butyrate as substrate in 0.1 M MOPS, 4 mM CaCl<sub>2</sub> pH 7.5 buffer. The activity was calculated in reference to a LIPOLASE™ standard curve generated by linear regression using concentrations of 1.0, 0.9, 0.8, 0.6, 0.4, 0.2 and 0.1 LU/ml.

6. For strain *F. venenatum* BBA 64537, fifty-one CAREZYME™ transformants were generated, of which fifteen were tested for expression in shake flasks, and sixty-seven LIPOLASE™ transformants were generated, of which eighteen were tested for expression in shake flasks. For strain *F. venenatum* ATCC 60879 twenty-nine CAREZYME™ transformants were generated, of which ten were tested for expression in shake flasks, and twenty-seven LIPOLASE™ transformants were generated, of which nine were tested for expression in shake flasks. Expression data for LIPOLASE™ and CAREZYME™ are expressed as percentages of the positive control values in Figures 3 a-c and 4 a-c.

Comparisons of expression data from *F. venenatum* ATCC 20334 and from the wild type strains *F. venenatum* BBA 64537 and *F. venenatum* ATCC 60879 are shown in Tables 1 and 2. The expression results for the two additional wild type strains compare favorably with the expression levels obtained with *F. venenatum* ATCC 20334. The results of t-tests, made using JMP software (SAS Institute, Inc., Cary, NC), are shown in Appendices C through F. The only significant ( $P < 0.01$ ) difference between enzyme yields of the two wild types studied in this report and ATCC 20334 was between *F. venenatum* ATCC 20334 and *F. venenatum* ATCC 60879 for CAREZYME™ (Appendix F). The average CAREZYME™ expression of transformants of the *F. venenatum* ATCC 60879 strain was significantly higher ( $P < 0.01$ ) than that for *F. venenatum* ATCC 20334.

The two additional wild type strains support the superiority of the *F. venenatum* species as an expression host.

7. I have developed a molecular method that is able to distinguish unambiguously *F. venenatum* from other *Fusarium* species (Yoder and Christianson, 1998, Species-specific Primers Resolve Members Of *Fusarium* Section *Fusarium*. Taxonomic Status of the Edible "Quorn" Fungus Re-evaluated. *Fungal Genetics & Biology* 23: 62-80). There are also morphological methods (Nirenberg, 1995, Morphological differentiation of *Fusarium sambucinum* Fuckel sensu stricto, *F. torulosum* (Berk. & Curt.) Nirenberg comb. nov. and *F. venenatum* Nirenberg sp.nov. *Mycopathologia* 129: 131-141) and molecular/phylogenetic and mycotoxin data (O'Donnell *et al.*, 1998, Molecular phylogenetic, morphological and mycotoxin

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data support re-identification of the Quorn fungus as *Fusarium venenatum*. *Fungal Genetics and Biology* 23: 57-67) which can be used to identify isolates of *Fusarium venenatum* and which have been proven to agree with our RAPD identification method.

Our identification method relies on sequence similarities (of bands amplified from genomic DNA) between isolates of proven identity and those of unknown identity. It simply involves running PCR reactions (using DNA from the unknown isolate(s) and our *F. venenatum* species-specific primers) and separating electrophoretically the amplified products on a gel. It is a robust method and positively identifies *F. venenatum* isolates both from different continents and different hosts. Our method does not require that cultures are in pristine condition nor does it require that the user must have extensive experience in fungal taxonomy (as do morphological identification methods). In addition it does not rely on expensive sequencing equipment or database searching and analysis, as do the molecular/phylogenetic methods.

Copies of each of these references (Yoder and Christianson, 1998, *supra*; Nirenberg, 1995, *supra*; and O'Donnell *et al.*, 1998, *supra*) are attached to this Declaration.

8. In conclusion, it is my opinion that the two additional wild type isolates of *F. venenatum* tested and described in this Declaration (see Appendices G, H, and I, respectively) are equivalent to or better than the *F. venenatum* strain ATCC 20334 in terms of transformation efficiency and heterologous protein production. It is also my conclusion that the *F. venenatum* strains are clearly superior to other *Fusarium* strains. For Examiner's reference, attached is Appendix J is the declaration filed in the parent case, patent application serial no. 08/816,915 which shows that *F. venenatum* is a superior host for heterologous protein production than *F. oxysporum* and *G. zeae*. Finally *F. venenatum* can be unambiguously distinguished from other *Fusarium* species.

9. The undersigned declarant declares further that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize any patent

Figure 1

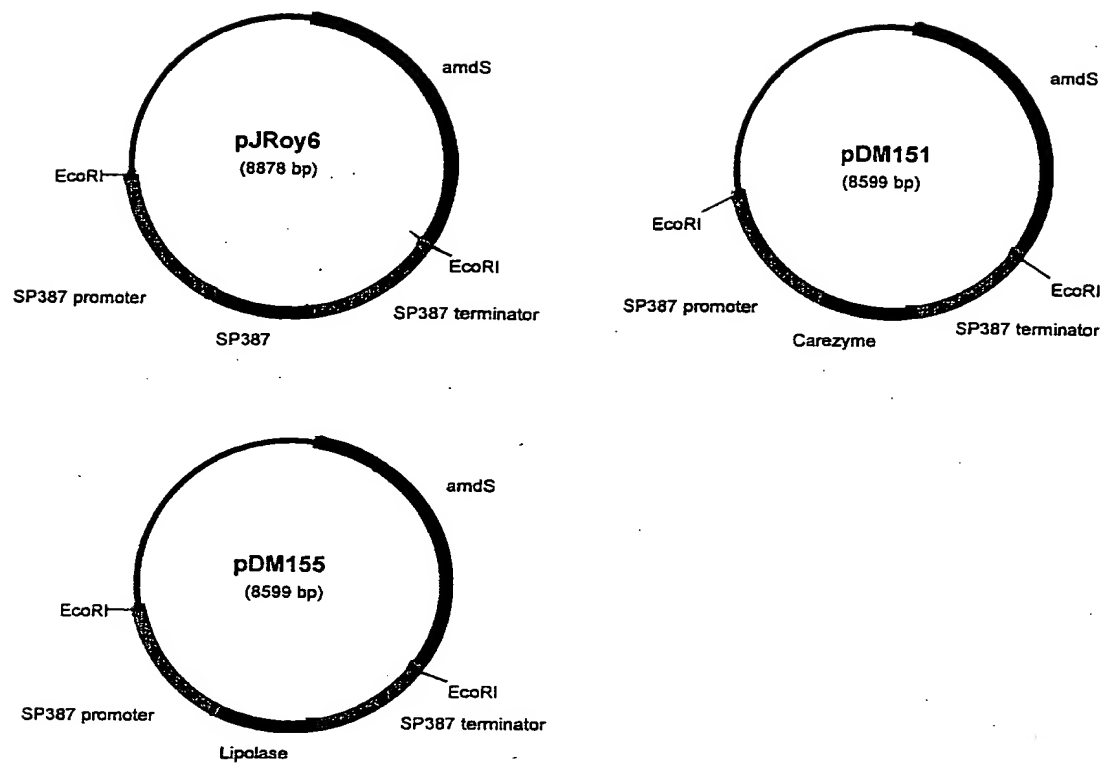
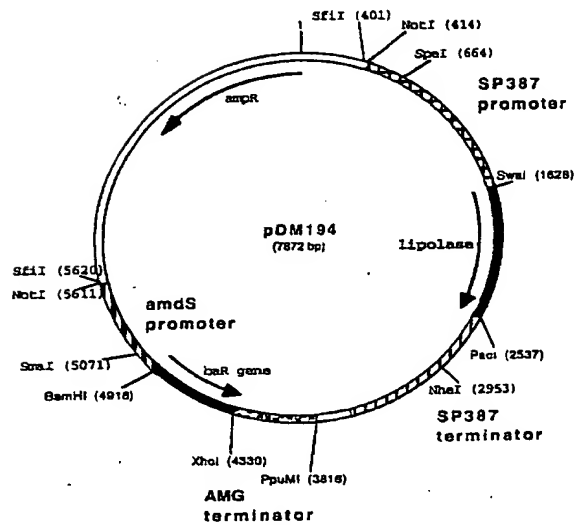
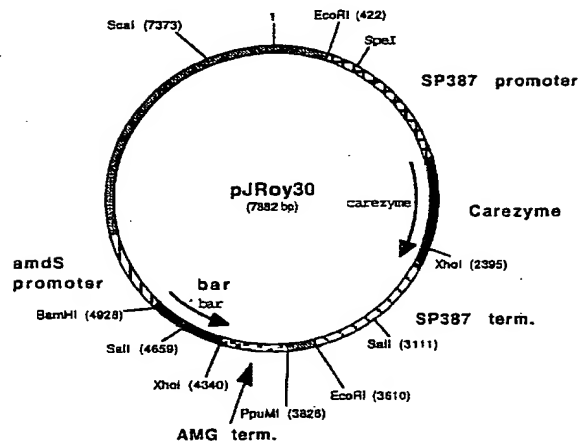
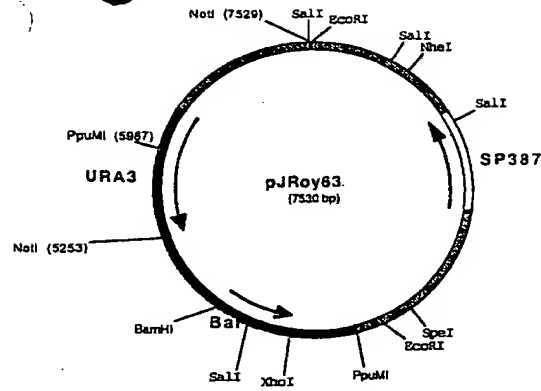
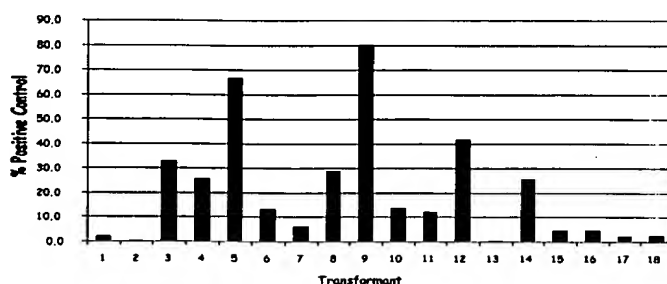


Figure 2

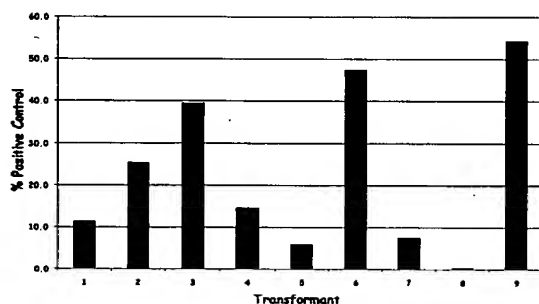




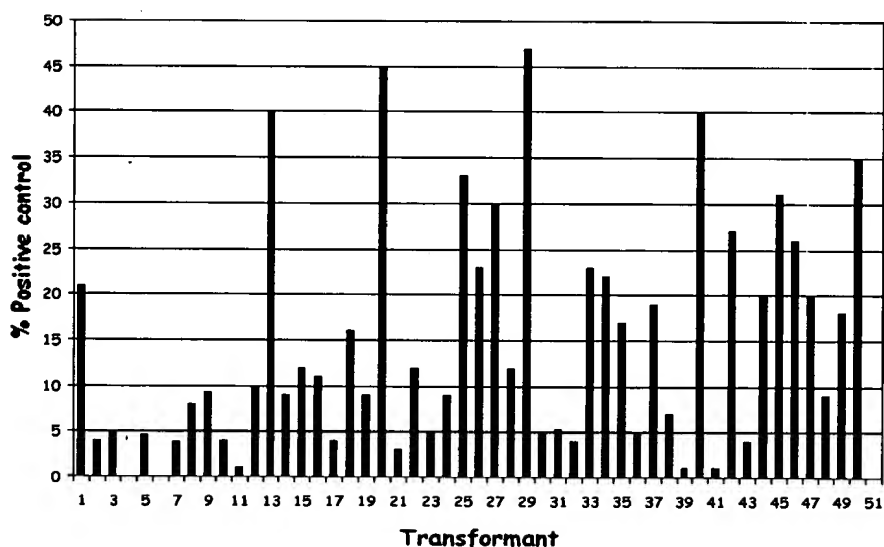
**Figure 3a.** Expression of LIPOLASE™ by *F. venenatum* BBA 64537 as a percentage of the positive control



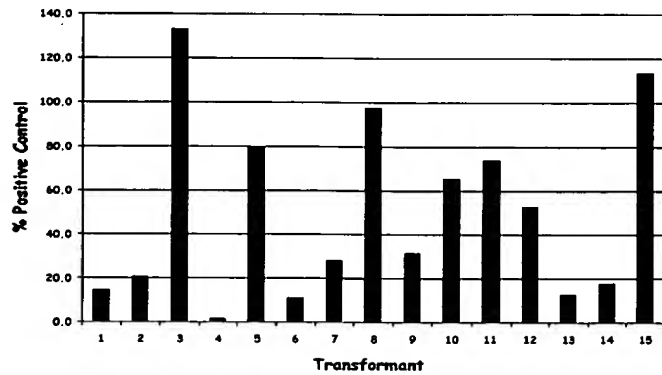
**Figure 3b.** Expression of LIPOLASE™ by *F. venenatum* ATCC 69879 as a percentage of the positive control



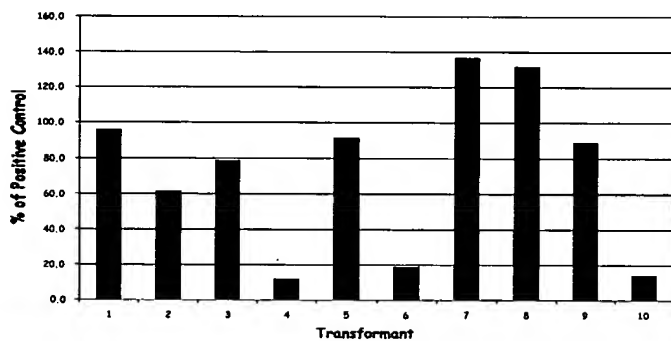
**Figure 3c.** LIPOLASE™ expression by *F. venenatum* ATCC 20334 as a percentage of the positive control



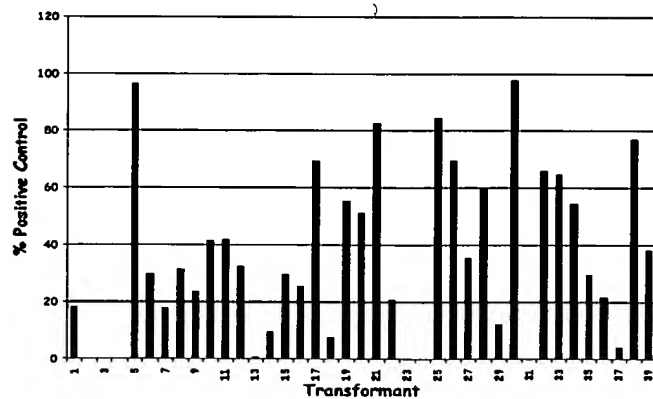
**Figure 4a.** CAREZYME™ expression by *F. venenatum* BBA 64537 as a percentage of the positive Control.



**Figure 4b.** CAREZYME™ expression by *F. venenatum* ATCC 60879 as a percentage of the positive Control.



**Figure 4c.** CAREZYME™ expression by *F. venenatum* ATCC 20334 as a percentage of the positive control.



**Table 1. Percentage of transformants expressing two heterologous enzymes at levels greater than or equal to 10% of the positive control.**

<i>F. venenatum</i> Strain	LIPOLASE™	CAREZYME™
ATCC 20334*	49 %*	74.4 %*
BBA 64537	55.5 %	93.3 %
ATCC60879	55.5 %	100 %

\* See Appendix J.

**Table 2. Percentage of transformants expressing two heterologous enzymes at levels greater than or equal to 50% of the positive control**

<i>F. venenatum</i> Strain	LIPOLASE™	CAREZYME™
ATCC 20334*	0 %*	44.8%*
BBA 64537	11.1 %	46.6 %
ATCC60879	11.1 %	70 %

\* See Appendix J.

**Appendix A.****LIPOLASE Assay Results**

Assay Detection Limit = &lt;1 LU/ml.

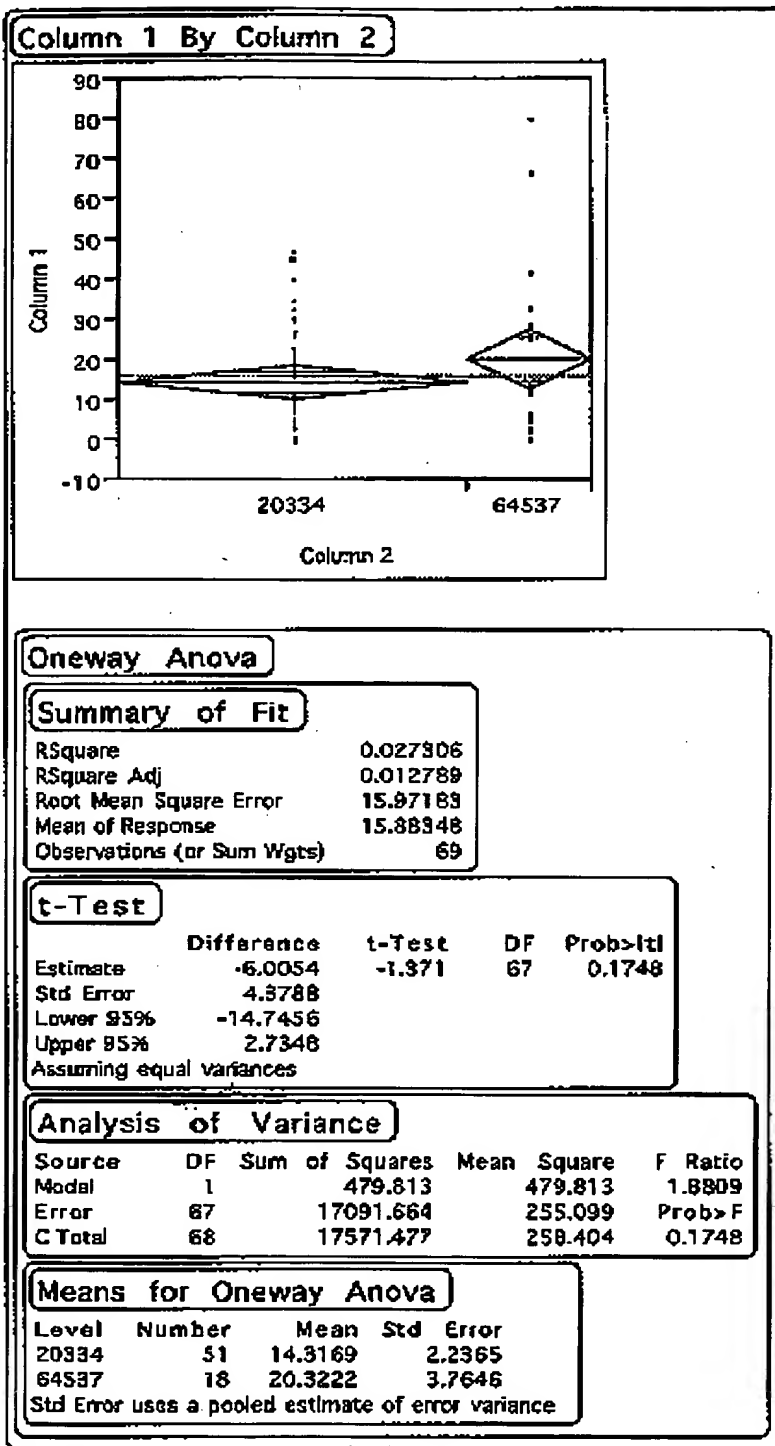
Strain	A LU/ml	B LU/ml	Mean LU/ml	Std Dev	% Positive Control
BBA 64537	0	0	0	0	0
ATCC 60879	0	0	0	0	0
DM192.19.2	4150	2815	3482.5	667.5	100
BBA 64537 Transformants					
1	78	77	77.5	0.5	2.2
2	9.4	9.3	9.35	0.05	0.3
3	1170	1125	1147.5	22.5	33.0
4	885	918	901.5	16.5	25.9
5	2371	2283	2327	44	66.8
6	471	455	463	8	13.3
7	229	221	225	4	6.5
8	998	1017	1007.5	9.5	28.9
9	2846	2743	2794.5	51.5	80.2
10	485	475	480	5	13.8
11	440	406	423	17	12.1
12	1430	1481	1455.5	25.5	41.8
13	9.9	9.5	9.7	0.2	0.3
14	895	895	895	0	25.7
15	171	172	171.5	0.5	4.9
16	166	168	167	1	4.8
17	88	87	87.5	0.5	2.5
18	95	98	96.5	1.5	2.8
ATCC 60879 Transformants					
19	273	519	396	123	11.4
20	829	932	880.5	51.5	25.3
21	1038	1718	1378	340	39.6
22	612	401	506.5	105.5	14.5
23	197	213	205	8	5.9
24	1498	1809	1653.5	155.5	47.5
25	162	363	262.5	100.5	7.5
26	20	2.7	11.35	8.65	0.3
27	1864	1917	1890.5	26.5	54.3

**Appendix B.**  
**CAREZYME Assay**  
**Results**

**Assay Detection Limit = <5 ECU/ml.**

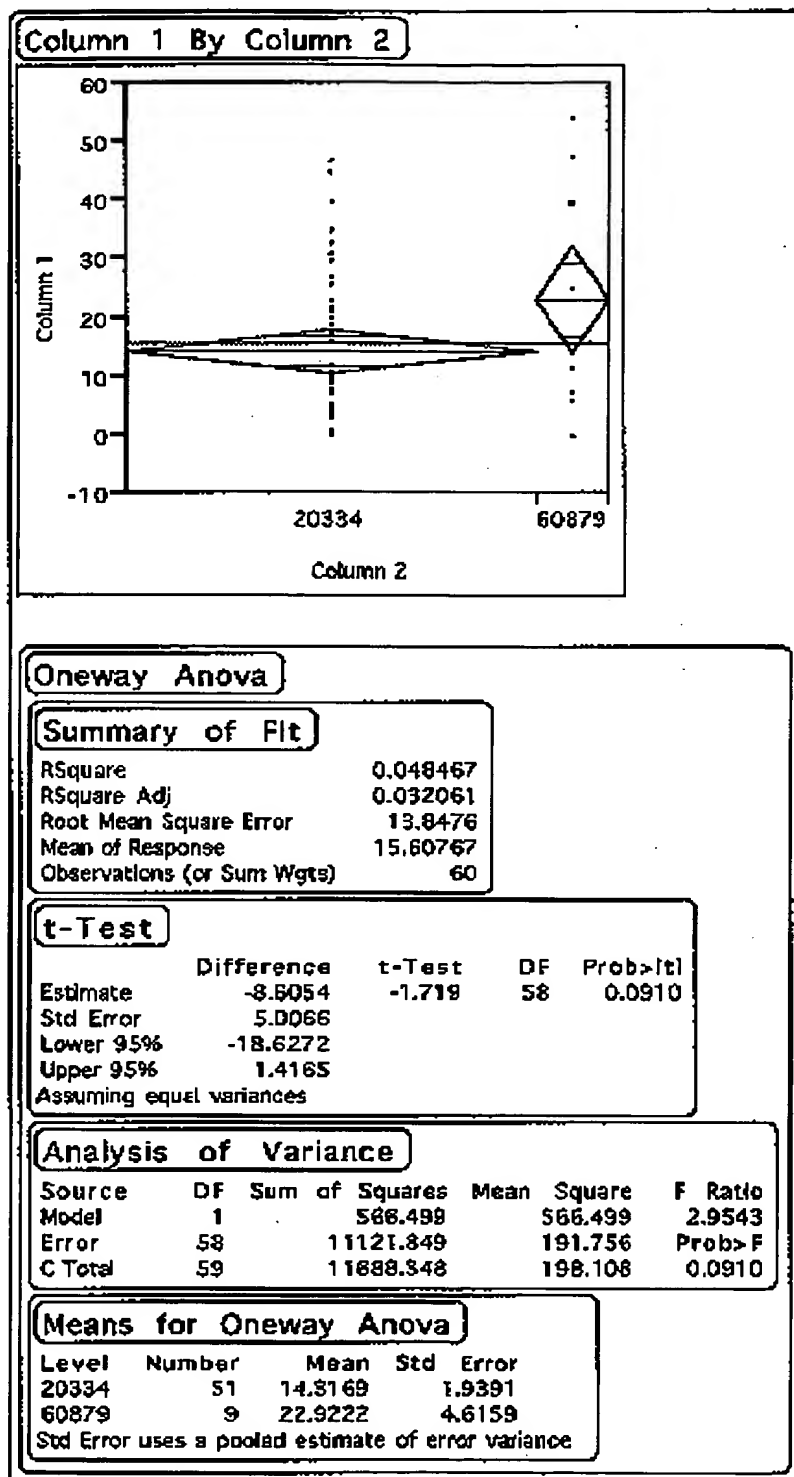
<b>Strain</b>	<b>A ECU/ml</b>	<b>B ECU/ml</b>	<b>Mean ECU/ml</b>	<b>Std Dev</b>	<b>% Positive Control</b>
BBA 64537	0	0	0	0	0
ATCC 60879	0	0	0	0	0
JRoy30.3	446	408	427	19	100
BBA 64537 Transformants					
31	73	52	62.5	10.5	14.6
32	71	105	88	17	20.6
33	518	617	567.5	49.5	132.9
34	4.3	10	7.15	2.85	1.7
35	361	320	340.5	20.5	79.7
36	46	49	47.5	1.5	11.1
37	127	113	120	7	28.1
38	408	422	415	7	97.2
39	137	133	135	2	31.6
40	286	271	278.5	7.5	65.2
41	332	298	315	17	73.8
42	223	226	224.5	1.5	52.6
43	42	65	53.5	11.5	12.5
44	98	52	75	23	17.6
45	471	498	484.5	13.5	113.5
ATCC 60879 Transformants					
46	467	352	409.5	57.5	95.9
47	194	329	261.5	67.5	61.2
48	325	345	335	10	78.5
49	53	48	50.5	2.5	11.8
50	437	341	389	48	91.1
51	95	66	80.5	14.5	18.9
52	663	505	584	79	136.8
53	604	521	562.5	41.5	131.7
54	400	359	379.5	20.5	88.9
55	62	57	59.5	2.5	13.9

Appendix C. Statistical Analysis: LIPOLASE™ – *F. venenatum* ATCC20334 vs

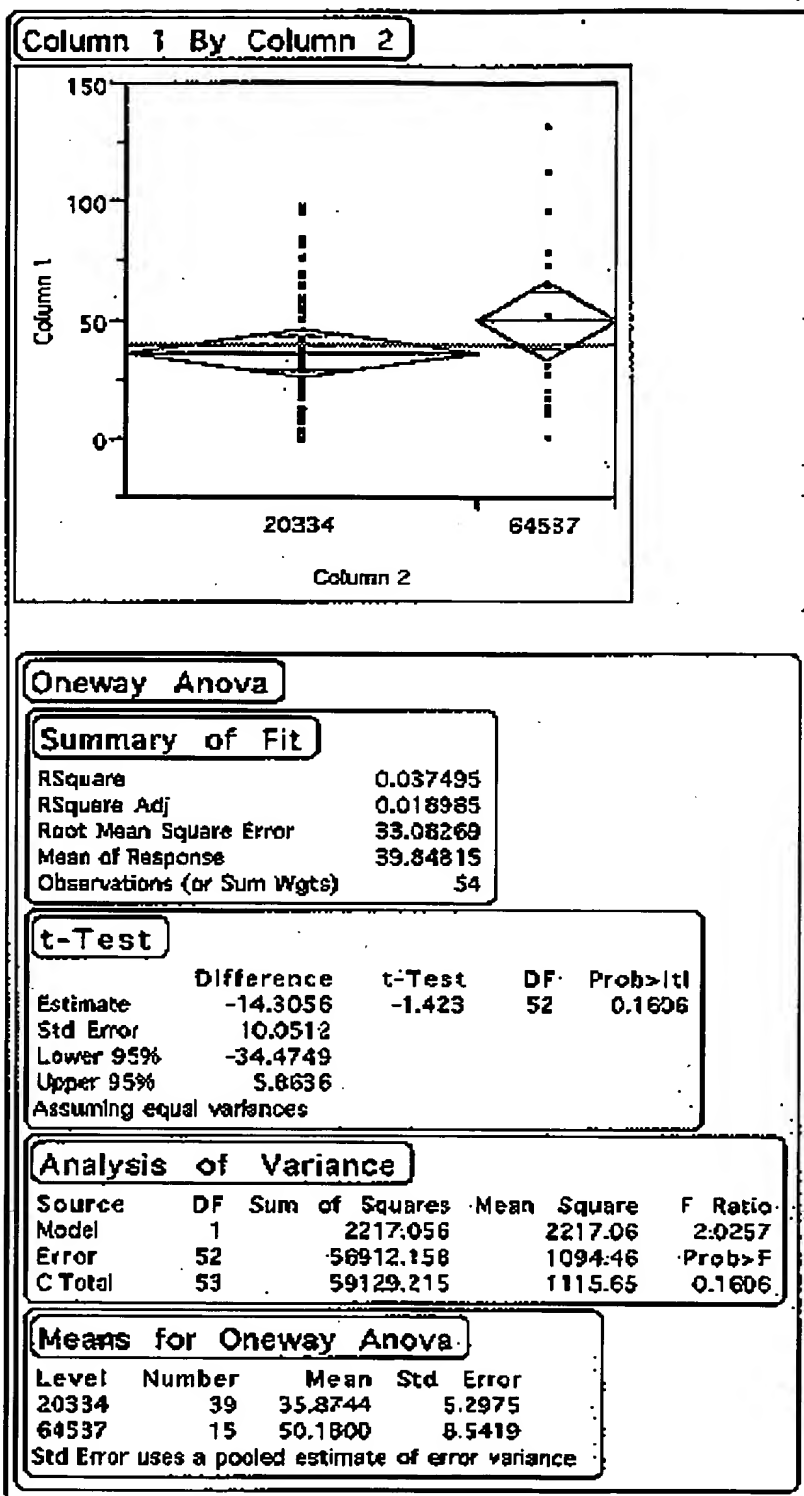


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Appendix D. Statistical Analysis: LIPOLASE™ – *F. venenatum* ATCC 20334 vs ATCC 60879

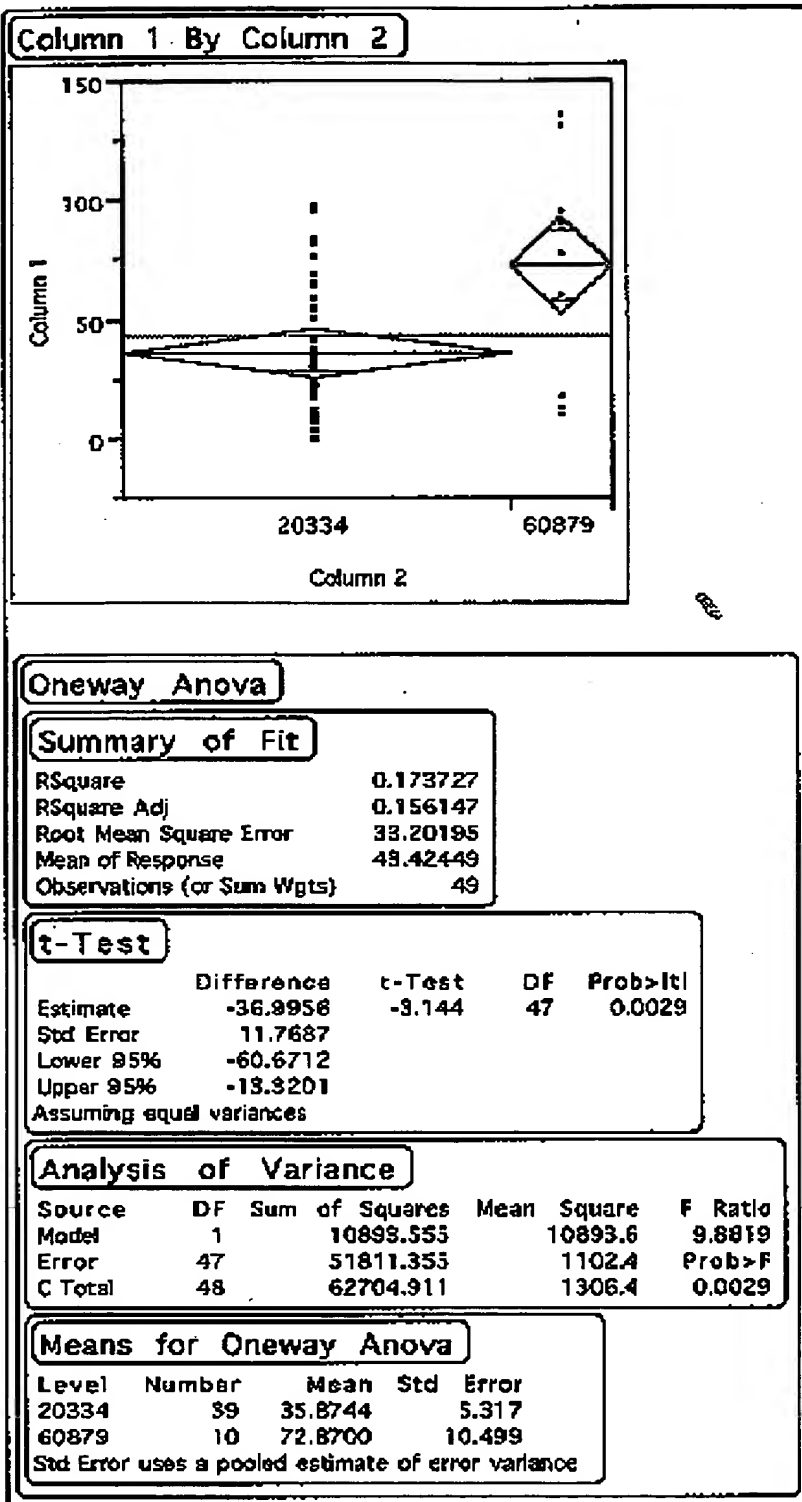


Appendix E. Statistical Analysis: CAREZYME™ – *F. venenatum* ATCC 20334 vs BBA 64537





# Appendix F. Statistical Analysis: Carezyme™ – ATCC20334 vs ATCC 60879



Attorney Docket No. 4216.240-US

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In re Application of: Royer *et al.*

Serial No.: 08/816,915

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Examiner: J. Railey

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**DECLARATION UNDER 37 C.F.R. § 1.132**

Assistant Commissioner for Patents  
Washington, DC 20231

Sir:

I, Wendy T. Yoder, do hereby state and declare that

1. I am one of the inventors of the subject matter disclosed and claimed in the above-captioned application.
2. I received a Ph.D. in Fungal Ecology and Genetics from the University of Bath, United Kingdom, in 1982. I have been employed at Novo Nordisk Biotech, Inc., Davis, California, since 1992 where I am currently a Senior Scientist.
3. I have read the Office Action dated March 29, 1999 issued in connection with the above-referenced Office Action and understand that claims 1-20 have been rejected over Blaiseu *et al.*, Dickman *et al.*, and Daboussi *et al.*, in view of Thrane and Towersey *et al.*
4. The experimental study and results described below were performed under my direct control and supervision using the following protocols.
5. In the experimental study, the *Fusarium* strains used were *F. venenatum* ATCC 20334 (=A 3/5), *F. oxysporum* (ATCC 34298) described by Daboussi *et al.* (U.S. Patent No. 5,446,138) and *Gibberella zeae* ATCC 20273 described by Dickman and Leslie (1992, *Mol. Gen. Genet.* 235: 458-462). Expression vectors were constructed according to Royer *et al.*, 1995, *Bio/Technology* 13: 1479-1483 containing the *Fusarium oxysporum* trypsin gene (SP387)

designated pJRoy6, *Humicola insolens* cellulase gene (CAREZYME™) designated pDM151, or *Thermomyces lanuginosus* lipase gene (LIPOLASE™) designated pDM155, using the *Fusarium oxysporum* trypsin gene (SP387) promoter and terminator for expression of the genes (U.S. Patent No. 5,837,847) and the *Aspergillus nidulans amdS* gene as the selectable marker. See Figure 1 for restriction maps of these vectors.

Protoplasts of *F. venenatum*, *F. oxysporum*, and *G. zeae* were prepared and transformed with the expression plasmids pDM153, pDM151, or pJRoy6 using from 1 to 20 µg per reaction according to the methods of Royer, 1995, *supra*. The total number of transformations made per strain per plasmid (pJRoy6, pDM151 and pDM155, respectively) were: *F. venenatum* ATCC 20334 = 99, 104, 120; *F. oxysporum* ATCC 34298 = 15, 29, 24; and *G. zeae* ATCC 20273 = 22, 32, 58.

A positive control strain was also run for each gene above. The control strains were *F. venenatum* JRoy63(10)7 containing pJRoy63 for SP387; *F. venenatum* JRoy30.3 containing pJRoy30 for CAREZYME™; and *F. venenatum* DM194.19.2 containing pDM194 for LIPOLASE™. Vectors pJRoy63, pJRoy30, and pDM194 contain the *Fusarium oxysporum* trypsin gene (SP387) promoter and terminator for expression of the genes and *bar* as the selectable marker. See Figure 2 for restriction maps of these vectors.

Agar plugs of each transformant were inoculated into 125 ml baffled shake flasks containing 25 ml of medium suitable for expression of the genes and incubated at 28°C and 200 rpm for 7 days. Centrifuged supernatants from each culture were assayed for enzyme activity as described below.

SP387 activity was determined at 405 nm using N-benzoylarginine-para-nitroanilide (BAPNA) as substrate in 0.1 M MOPS, 4 mM CaCl<sub>2</sub> pH 7.5 buffer at 30°C. The activity was calculated in reference to a SP387 standard curve generated by linear regression using concentrations of 0.244, 0.217, 0.163, 0.108, and 0.0542 U/ml.

CAREZYME™ activity was determined at 600 nm using 2% azo-carboxymethylcellulose as substrate in 100 mM MOPS pH 7.0 buffer at 45°C for 30 minutes. The activity was calculated in reference to a CAREZYME™ standard curve generated by linear regression using concentrations of 20, 15, 10, 5 and 2.5 ECU/ml.

LIPOLASE™ activity was determined at 405 nm using p-nitrophenyl butyrate as substrate in 0.1 M MOPS, 4 mM CaCl<sub>2</sub> pH 7.5 buffer. The activity was calculated in reference to a LIPOLASE™ standard curve generated by linear regression using concentrations of 1.0, 0.9, 0.8, 0.6, 0.4, 0.2 and 0.1 LU/ml.

6. The process of identifying robust production strains for the commercial production of heterologous proteins involves screening large numbers of potentially high yielding transformants of a suitable host strain. There are three basic criteria required for a host cell to be

useful in identifying potential production strains.

- (1) The host strain should provide good, consistent transformation efficiencies with different genes so the strain is reliable as a host for expressing a wide range of genes from different microorganisms.
- (2) Transformation of the host strain with a gene should yield a high percentage of transformants expressing each gene.
- (3) A sub-population of the transformants of the host strain should express each of several different classes of enzymes in relatively high amounts.

It is very important that the host strain satisfy all three criteria for the strain to be useful as a host for expressing a wide range of genes because it is traditionally necessary to screen a very large number of high yielding transformants for the enzyme activity of interest before a potential production strain can be identified. Generating a large number of transformants is necessary because transformation of the host cell with a heterologous gene is a mutagenic process where other, unpredictable and uncharacterized, alterations may be introduced into the host genome and which may affect, for example, mycelial morphology, growth rate, sporulation, protease production or the capacity of a strain for fermentor scale-up. In addition, transformants are stable to varying degrees (due to reductions in copy number of the selectable marker and heterologous gene, variations in the site of integration of the heterologous DNA, and for other reasons, which are not well understood at this time). These other properties only become evident during scaled-up growth in fermentors and it is not uncommon for a primary candidate (selected as being the highest expressing transformant in shake flasks) to fail in scale-up.

7. The experimental study compared *F. venenatum* as a host strain with *F. oxysporum* and *G. zeae* with respect to these three criteria.

Criterion (1). The host strain should provide good, consistent transformation efficiencies with different genes so the strain is reliable as a host for expressing a wide range of genes from different microorganisms.

Figure 3 shows the level of expression of the transformants relative to the positive controls. As shown therein, all three strains yielded transformants capable of expressing the three enzymes. However, many more transformants of *F. venenatum* were generated for all three genes. Indeed, the number of *F. venenatum* transformants were so large that all were not assayed. In contrast, the number of transformants of *F. oxysporum* and *G. zeae* were not large and, thus, all were assayed.

Moreover, only the *F. venenatum* transformants consistently produced significant levels of all three heterologous enzymes. In contrast, the majority of the *F. oxysporum* transformants produced low or undetectable levels of all three enzymes. Additionally, while a reasonable

number of *G. zeae* transformants producing LIPOLASE™ were generated, only four CAREZYME™ transformants were obtained with this species from 32 transformation reactions.

Criterion 2. Transformation of the host strain with a gene should yield a high percentage of transformants expressing each gene.

When developing a production strain, a high number of transformants expressing the enzyme must be screened to identify those with the best potential to perform well in fermentors. To determine the overall potential of these *Fusarium* strains to give rise to sufficient numbers of expressing transformants, a comparison of the percentage of transformants that yielded levels greater than or equal to 10% of the positive control is shown in Tables 1 and 2. *F. venenatum* had the highest percentages for both SP387 and CAREZYME™ and almost half of the *F. venenatum* transformants produced significant amounts of LIPOLASE™. While 65% of the *G. zeae* LIPOLASE™ transformants produced greater than 10% of the control, this species did not perform well at yielding large numbers of transformants expressing greater than 10% of the control for SP387 or CAREZYME™.

**Table 1. Percent of transformants expressing 3 heterologous enzymes at levels greater than or equal to 10% of the positive control<sup>a</sup>**

Strain	SP387	CAREZYME™	LIPOLASE™
<i>F. venenatum</i>	82.1%	74.4%	49.0%
<i>F. oxysporum</i>	31.6%	12.9%	3.7%
<i>G. zeae</i>	38.5%	25.0%	65.2%

<sup>a</sup> Total number of transformants assayed for SP387, CAREZYME™, and LIPOLASE™, respectively, are *F. venenatum* n = 28, 39, 51; *F. oxysporum* n = 19, 31, 27; *G. zeae* n = 13, 4, 23. 39 additional CAREZYME™ and 96 additional LIPOLASE™ *F. venenatum* transformants were generated, but not assayed.

**Table 2. Ratio of *F. venenatum* expression relative to that of *F. oxysporum* and *G. zeae* for three heterologous enzymes<sup>b</sup>**

Strain	SP387	CAREZYME™	LIPOLASE™
<i>F. oxysporum</i>	2.6	5.8	13.2
<i>G. zeae</i>	2.1	3.0	0.75

<sup>b</sup> Based on Table 1 and calculated by dividing the percentage of *F. venenatum* transformants expressing greater than or equal to 10% of the positive control by the equivalent number for *F. oxysporum* and *G. zeae*.

Criterion 3. A sub-population of the transformants of the host strain should express each of several different classes of enzymes in relatively high amounts.

For two out of the three enzymes (SP387 and CAREZYME™), *F. venenatum* also gave rise to the greatest percentages of transformants expressing more than 50% of the positive control (Table 3), indicating its potential to give rise to high-yielding strains.

**Table 3. Percent of transformants expressing 3 heterologous enzymes at levels greater than 50% of the positive control<sup>a</sup>**

Strain	SP387	CAREZYME™	LIPOLASE™
<i>F. venenatum</i>	10.7%	44.8%	0%
<i>F. oxysporum</i>	0%	0%	0%
<i>G. zeae</i>	0%	25.0%	13.0%

<sup>a</sup> Total number of transformants assayed for SP387, CAREZYME™, and LIPOLASE™, respectively, are *F. venenatum* n = 28, 39, 51; *F. oxysporum* n = 19, 31, 27; *G. zeae* n = 13, 4, 23. 39 additional CAREZYME™ and 96 additional LIPOLASE™ *F. venenatum* transformants were generated, but not assayed.

8. The data indicate clearly that *F. oxysporum* does not qualify as a potential expression host for heterologous enzymes, when compared to *F. venenatum*, since the former does not satisfy criteria (2) and (3) for any of the three genes assayed.

The data also indicate that *G. zeae* does not satisfy the above criteria. While it is true for one of the three genes, i.e., that encoding LIPOLASE™, a reasonable number of high expressing transformants were obtained, CAREZYME™ transformants of *G. zeae* were very difficult to generate and only one of the four *G. zeae* transformants obtained from 32 transformation reactions expressed any heterologous protein. This species, therefore, cannot be considered sufficiently robust or consistent to meet the three criteria outlined above to qualify as a potential production host.

9. As shown above, *F. venenatum* is the only host strain that satisfies all three criteria. It is my opinion that *F. venenatum* is a superior host for heterologous protein production than *F. oxysporum* and *G. zeae* for the following reasons: (1) *F. venenatum* enables consistently good transformation efficiencies with a broad spectrum of heterologous genes; (2) a high proportion of the *F. venenatum* transformants express the genes; and (3) a sub-population of the *F. venenatum* transformants in (2) express the genes in high amounts.

10. The undersigned declarant declares further that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize any patent issuing thereon.

Signed this 29 day of September 1999

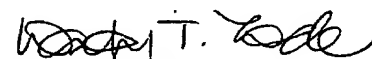
  
 Wendy T. Yoder

Figure 1

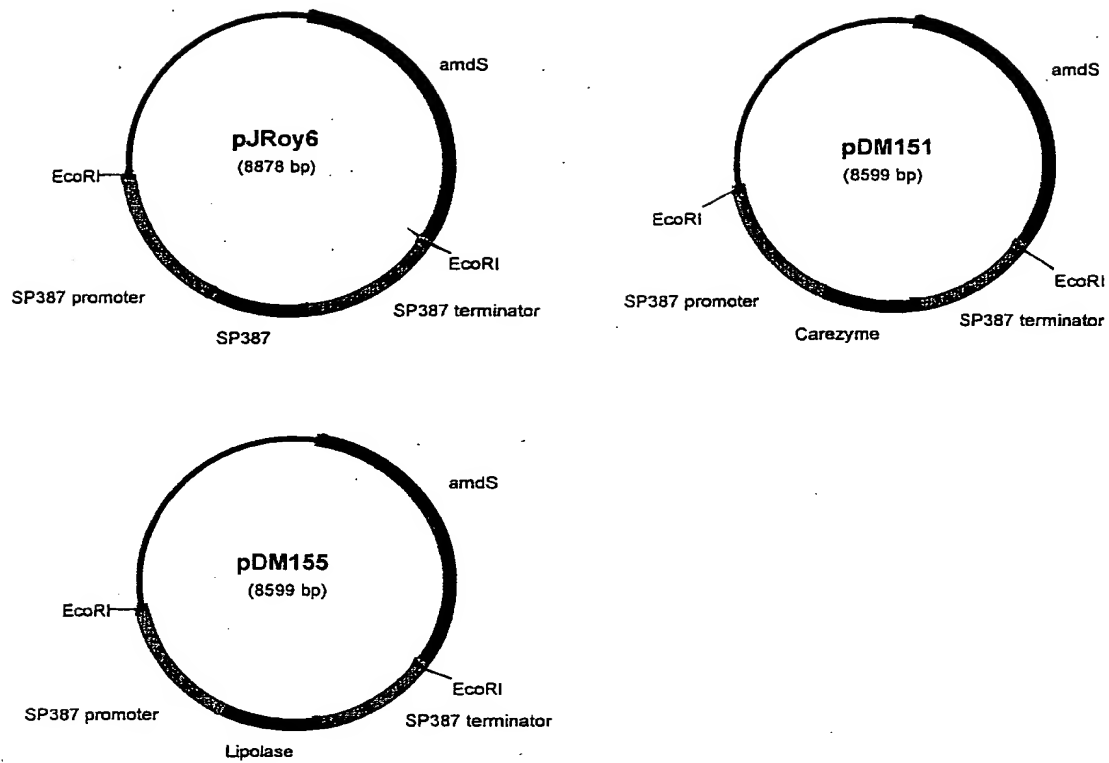


Figure 2

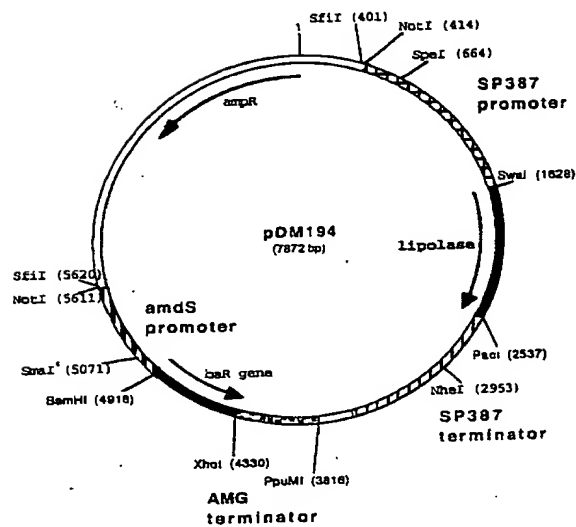
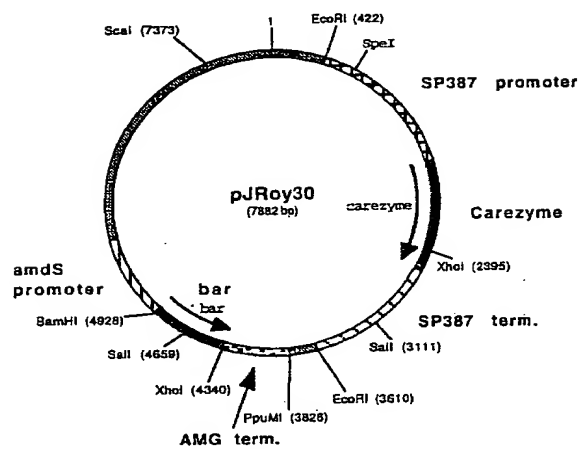
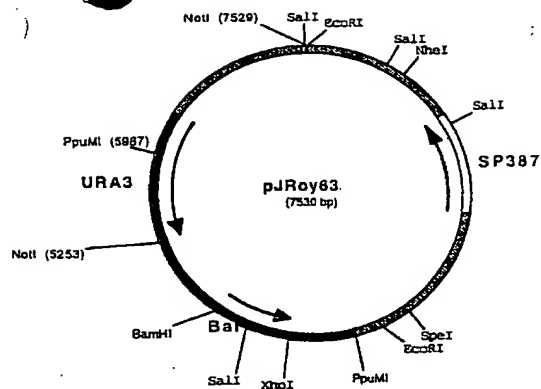




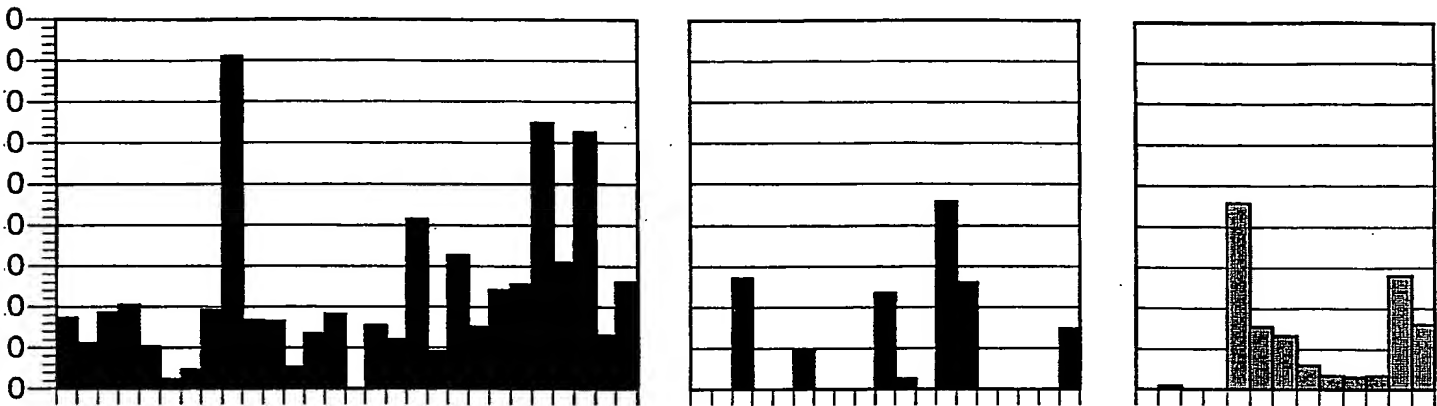
Figure 3

SP387

*F. venenatum*

*F. oxysporum*

*G. zeae*

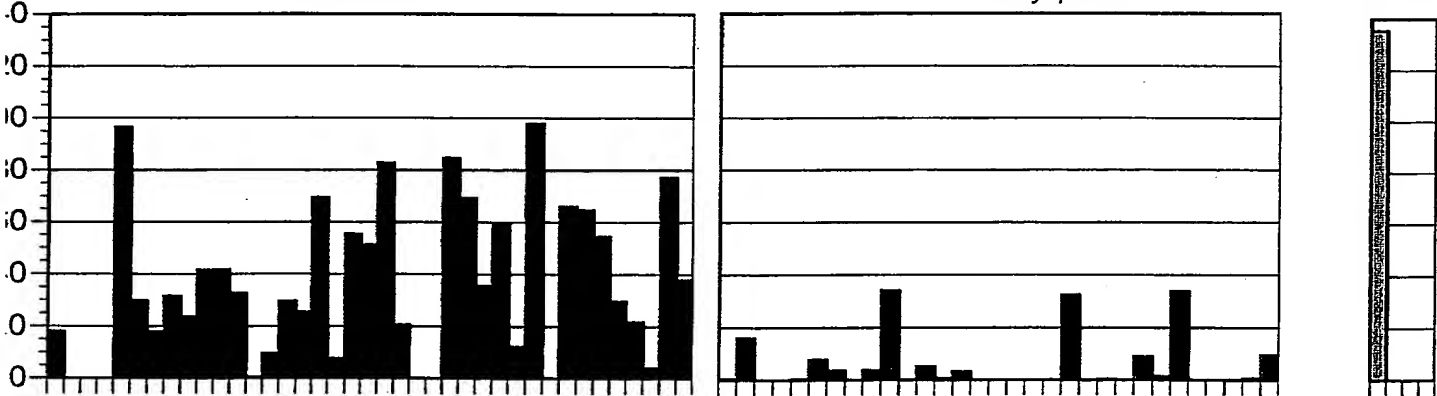


Carezyme

*F. venenatum*

*F. oxysporum*

*G. zeae*

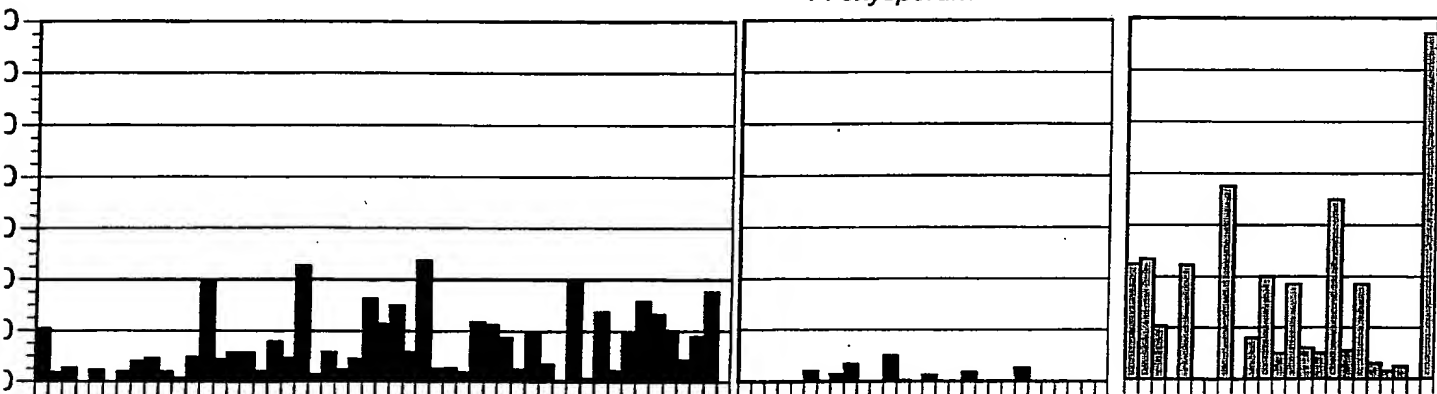


Lipolase

*F. venenatum*

*F. oxysporum*

*G. zeae*





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